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The interaction between growth factor and steroid hormone receptors may play a critical role in the breast cancer progression. The principle objective of this proposal is to elucidate the *in vivo* importance of cross-talk between estrogen and ErbB-2 signaling pathways. We first plan to establish transgenic mice that express both an constitutively active estrogen receptor and its co-activator in the mammary epithelium. To accomplish this objective, we will derives eparate strains of transgenic mice that carrying either a constitutively activated ER or its co-activatorAIB1 under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter enhancer. Once these transgenic strains have been derived we will assess whether activation of estrogen signaling pathway can either positively or negatively influence ErbB-2 mediated tumor progression by interbreeding these strains to separate strains of transgenic mice expressing an activated erbB-2 oncogene in the mammary epithelium. Because activation of estrogen signaling pathway is known to influence the transcriptional activity of the erbB-2 promoter, we plan to use a unique ErbB-2 mammary tumor model in which mammary epithelial expression of the ErbB-2 oncogene is under the transcriptional control of the endogenous ErbB-2 promoter. The results of these studies will provide important insight into these crucial signaling pathways in breast cancer progression.

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Introduction

The induction of human breast cancer involves the complex interplay of hormonal, and genetic events. There is an extensive body of literature implicating the activation of ERα in induction of sporadic breast cancer. Despite the importance of this signaling pathway, there are relative few studies that have directly assessed the ERα signaling axis in tumor progression. Another important genetic event in sporadic breast cancer is the amplification and consequent overexpression of erbB-2 gene (7, 8). Although amplification and elevated expression of erbB-2 has been established as an important event in sporadic breast cancer, comparatively little is known concerning the interaction of erbB-2 and ERα in sporadic breast cancer.

Activation of receptor tyrosine kinases such as erbB-2 can lead to the stimulation of downstream signaling pathways such as the MAP kinase pathway. In turn activated MAP kinase can result in the direct phosphorylation of ERα and stimulation of its transcriptional activity (3). Additional evidence supporting the concept that erbB-2 signaling may influence ERα function stems from the recent observation that c-Src and ERα can form specific complexes that result in the activation of the MAP kinase cascade (4). Because activation of the c-Src kinase appears to be an important signaling pathway in ErbB-2 mammary tumor progression (4-6), c-Src may play an important role in the crosstalk between ERα and ErbB-2 receptors.

Although the data reviewed above argue that complex interaction exists between ERα and ErbB-2 signaling, comparatively little is known concerning this interaction in mammary tumor progression. The principle focus of this application is to explore the significance of the ERα signaling axis in ErbB-2 mammary tumor progression. To accomplish this, we have derived separate strains of transgenic mice that express a constitutively activated ERα or its cognate co-activator AIB1 in the mammary epithelium. To assess the consequences of the activation of ERα signaling pathway on ErbB-2 induced mammary tumor progression, we plan to interbreed these strains to a transgenic mouse model that expresses an activated erbB-2 under the transcriptional control of endogenous erbB-2 promoter.

Body

Establishment of transgenic mice that express a constitutively activated ERα in the mammary epithelium.

Given the potential importance of ERα in sporadic breast cancer, one of our first goals was to generate transgenic mice that express an activated version of ERα in mammary epithelium. The activated version of ERα possesses an asparagine substitution at tyrosine 537 that mimics a ligand activated ERα (9). To accomplish this, a cDNA encoding the activated ERα was cloned into an MMTV based expression plasmid (Figure 1A) and microinjected into FVB/N one cell mouse embryos. A total of eight founder animals were generated.

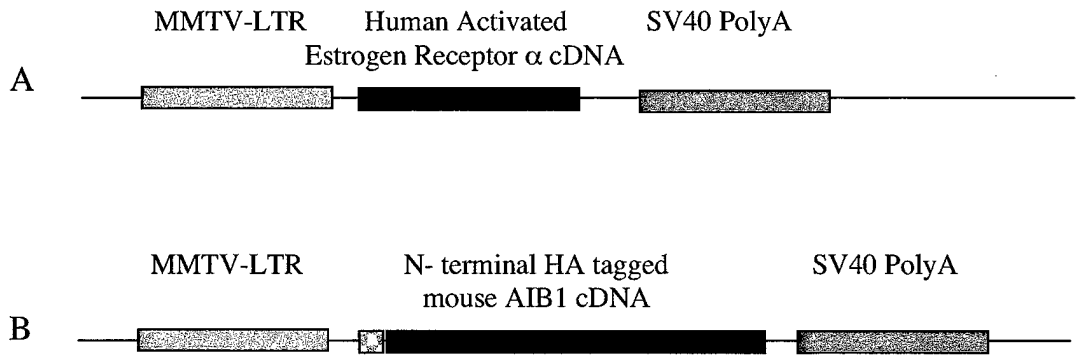

Figure 1 : Schematic representations of the MMTV-activated ERα (A) and MMTV- AIB1 (B).

To assess whether transgenic strains were expressing the transgene in the mammary epithelium, RNA was extracted from either virgin or lactating mammary epithelium from the various progeny and quantitative real time RT-PCR was performed on RNA samples with primers directed to the SV40 component of the transgene. The results revealed that of the eight founder strains tested, three of the strains expressed detectable levels of the activated ER transgene (Figure 2). In all three cases the lactating epithelial sample expressed significantly higher levels of the MMTV/ERα transgene. In particular the 02-351-5 strain expressed 8 fold elevated levels of ERα transgene compared to the two other expressing strains.

- 3 founders express MMTV-activated ERalpha :
  - 02-351-5 (virgin and lactating mammary gland)
  - 02-347-2 (lactating mammary gland)
  - 02-346-1 (lactating mammary gland)

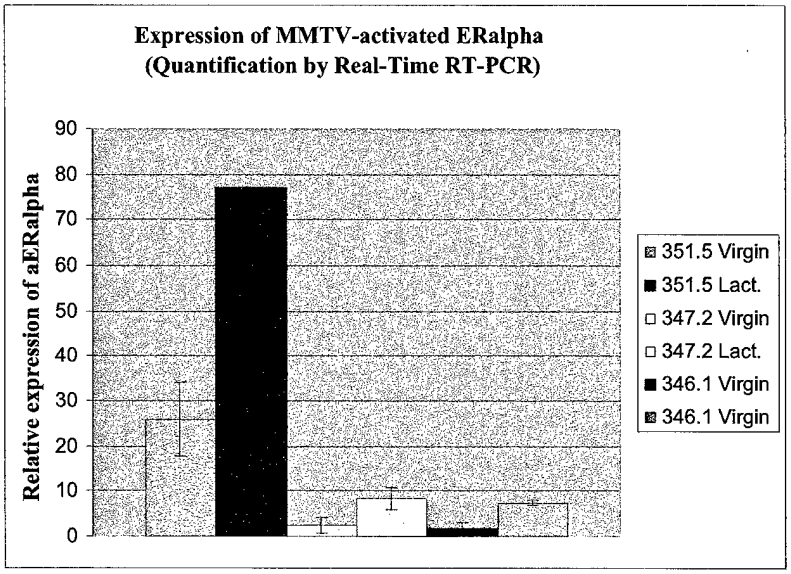

Figure 2 : Quantification of MMTV-activated ERα expression by Real-Time RT-PCR using primers directed to the SV40 component of the transgene.

To explore whether ectopic expression of ERα interfered with normal mammary gland development, we performed wholemount and histological analyses on 5 months old virgin mammary epithelium derived from the 02-351-5 founder strain. The results showed that despite the elevated expression of activated ERα observed in the mammary epithelium of these transgenic strains, both wholemount and histological analyses of mammary glands revealed no overt signs of hyperplasia (Figure 3). These observations suggest that at least at these stages of virgin mammary development , ectopic expression of activated ERα is not associated with any obvious mammary gland abnormalities. However wholemount examination of lactating mammary glands revealed a potential defect in lactating glands.

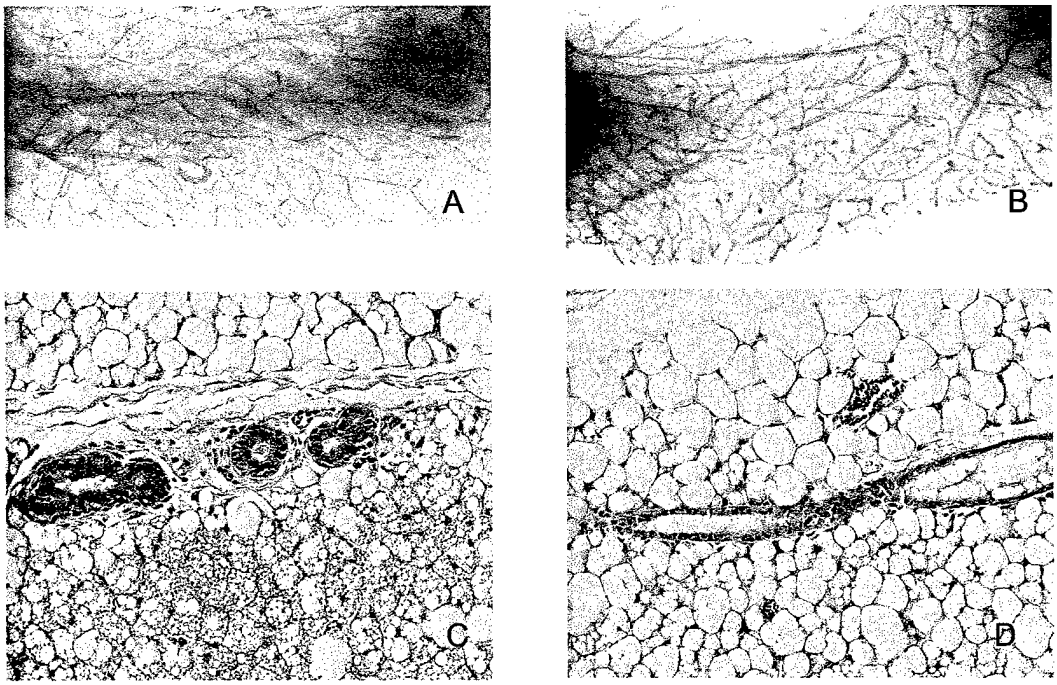

Figure 3 : Whole mount and histological analysis of virgin mammary glands. (A, B) Whole mount preparations of virgin glands from 5 months old control (A) and MMTV-activated ERα expressing (B) mice. (C, D) H&E stained sections of virgin glands from 5 months old control (C) and MMTV-activated ERα expressing (D) mice.

Although lobular alveolar development and milk production could be detected in the transgenic mammary glands (Figure 4C), closer examination of the wholemounts revealed regions of delayed alveolar development compared to wild type controls (compare Figure 4B to Figure 4D). These observations suggest that ectopic expression of ERα may impact on the latter stages of mammary differentiation.

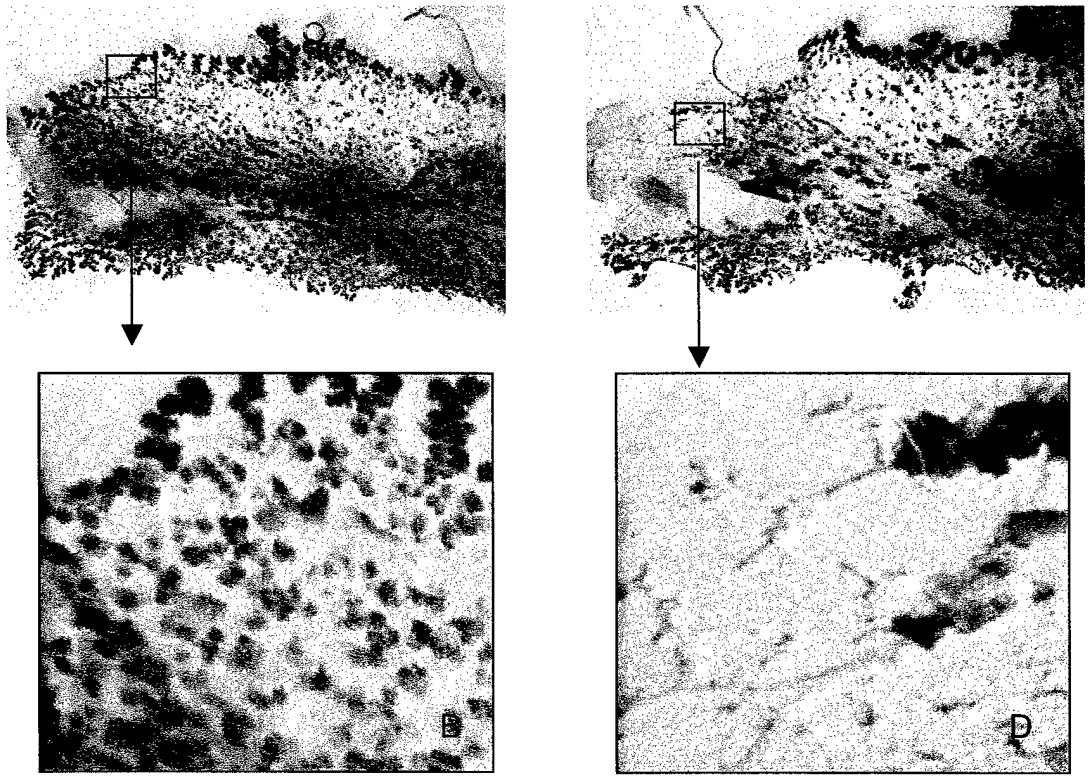

Figure 4 : Whole mount preparations of lactating mammary glands from first day post-partum control (A,B) and MMTV-activated ERα expressing (C,D) mice. Mice used in this experiment were age-matched and uniparous.

To confirm that expression of the activated ERα in the mammary epithelium was confined to the mammary epithelial cells, we also performed immunohistochemical staining on virgin mammary glands from the ERα expressing strain. Because the antibodies were directed to the human ERα and do not cross react with the endogenous mouse ERα receptor, we can specifically detect expression of transgene driven in ERα in histological sections. Examination of virgin glands revealed uniform staining of ERα in the transgenic virgin mammary gland (Figure 5C and 5D). By contrast examination of control virgin gland failed to reveal any ERα positive cells (Figure 5A and 5B). In addition to the observed uniform and elevated expression of ERα, higher magnification of the transgenic mammary glands revealed that activated ERα was localized primary to its expected nuclear localization (Inset , Figure 5D).

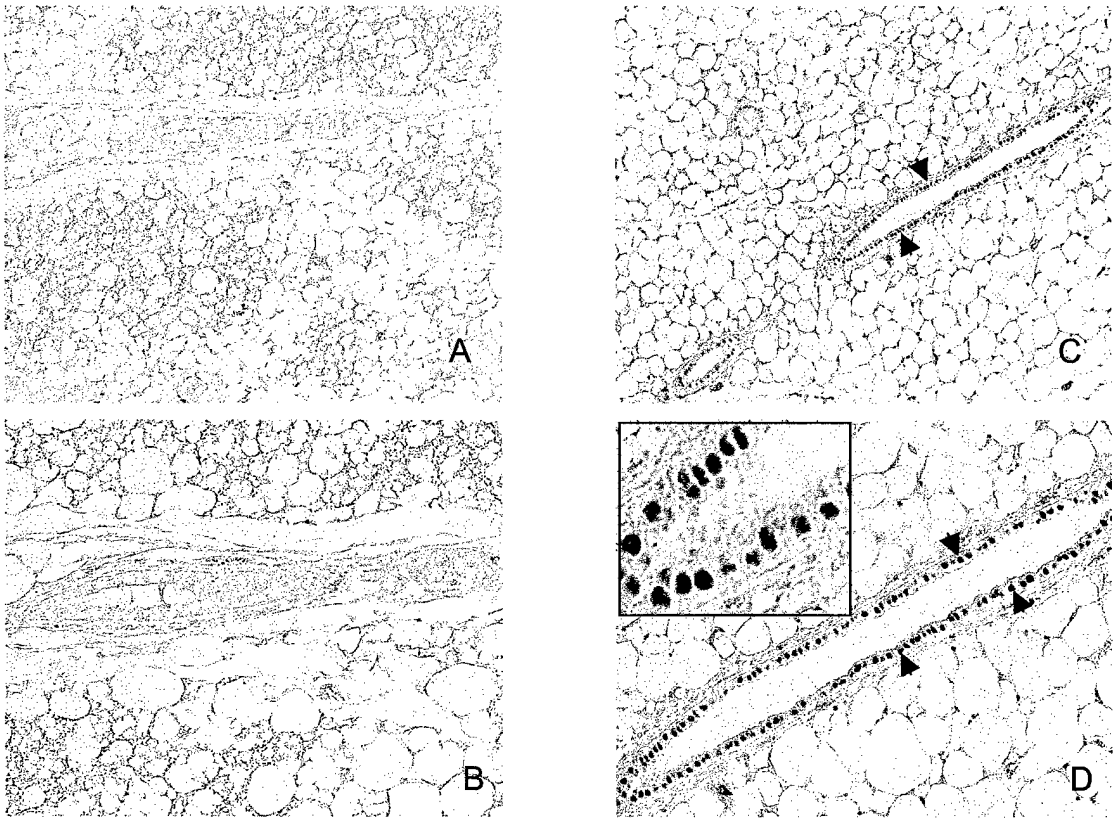

Figure 5 : Immunostaining of ERα on paraffin embedded sections of virgin mammary glands from 5 month old control (A,B) and MMTV-activated ERα expressing (C,D) mice. The sections were stained with a mouse monoclonal antibody specific for human estrogen receptor alpha (Novocastra, NCL-ER-6F11) followed by a goat anti-mouse HRP labeled secondary antibody. Inset in D shows a higher magnification of ERα nuclear staining. Representative cells exhibiting nuclear staining for ERα are indicated by black arrows.

Given the elevated and mammary specific expression of ERα observed in this MMTV/ERα transgenic strains, we have now generated a cohort of 20 virgin female transgene carriers that will be monitored for the occurrence of mammary tumors over the next year. In addition we have also initiated interbreeding of this MMTV/ERα strain with separate strain of transgenic mice expressing a Cre inducible activated erbB-2 under its endogenous promoter (2). Once the appropriate cohort of female animal co-expressing activated erbB-2 and ERα have generated, we will monitor these mice for the occurrence of mammary tumors. This set of experiments should allow us to ascertain whether ectopic expression of activated ERα can impact on ErbB-2 induced mammary tumor progression.

Another important goal of this research program was to generate transgenic mice that ectopically express the ERα coactivator AIB1. To this end, we have placed an HA tagged AIB1 cDNA under the transcriptional control of the MMTVpromoter (Figure 1B). Preliminary expression analyses of the mammary epithelium derived from MMTV/AIB founders have failed to detect any founder strains that express elevated levels of AIB. We

are in the process of generating further founder strains. Once we identified an appropriate transgene expressing strain, we will initiate interbreeding of this MMTV/AIB strain with separate MMTV/ERα and activated erbB-2 strains.

KEY RESEARCH ACCOMPLISHMENTS

1. Generation of transgenic mice expressing elevated levels activated ERα in the mammary epithelium
2. Identification of potential alveolar differentiation defect in MMTV/ERα strains
3. Generation of a cohort of virgin MMTV/ERα mice that will be monitored for tumor formation.

CONCLUSIONS

We have made substantial progress on DOD sponsored research program designed to investigate the interaction of ERα and ErBb-2 signaling pathways in mammary tumor progression. Firstly we have successfully established three independent transgenic strains that ectopically express ERα in the mammary epithelium. Using immunohistochemical staining approach we have further demonstrated that ERα is uniformly expressed in the mammary epithelium. Despite the ectopic expression of activated ERα wholemount analyses of virgin glands failed to exhibit any of evidence hyperplastic expansion. Indeed consistent with this observation, it has been reported that in normal human breast epithelium, estrogen receptor positive cell fail to proliferate (1). To confirm whether the estrogen receptor positive epithelium in transgenic strains also exhibit this proliferation block, we will perform immunohistochemical staining on these glands with a Ki67 proliferative marker. This set of experiments should allow us to assess whether, like in human breast cancer epithelial cells, activation of ERα induces a proliferative block. Should female transgenic mice eventually develop mammary tumors, we will utilize both comparative genome hybridization and gene expression profiling approaches to determine if ERα induced tumors have undergone genetic alteration that have enable them to escape this proliferative block associated with expression of ERα. Given the high levels of ERα expression observed in this strain, we will monitor the cohort of female mice from this strain for mammary tumor development. In addition, we have initiated crosses of this strain with Cre-inducible activated erbB-2 strains to explore whether elevated expression of activated ERα can influence ErbB-2 induced tumorigenesis in this transgenic mouse of human breast cancer.

Another observation that we will explore further is the aberrant alveolar differentiation observed in the MMTV/ERα strains. To explore whether ectopic expression of activated ERα can interfere with normal mammary differentiation, we will conduct wholemount and histological analyses of the mammary glands from day 5, 10 and 15 pregnant mice. To complement these histological approaches, we plan to isolate mammary gland RNA

from the contralateral fat pads and test for the expression of mammary epithelial differentiation markers such as β-casein and WAP proteins. These detailed molecular analyses should provide important insight into the potential role of ERα in mammary differentiation.

Our initial screen of MMTV/AIB founder strains as yet to reveal an adequate transgene expressor. One potential explanation for the lack of transgene expressors is that the transgene has integrated in an area of condensed chromatin. Indeed it is our experience that we have to generate 10 lines to obtain several expressing strains. Consequently, we are in the process of generating additional strains through additional rounds of microinjection. We will hopefully obtain the appropriate MMTV/AIB strains within the next few months.

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